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Ramularia hydrangeicola sp. nov. with distinctive traits on *Hydrangea serrata* f. *acuminata* in Korea

Ji-Hyun Park & Hyeon-Dong Shin*

Division of Environmental Science and Ecological Engineering, Korea University, Seoul 02841, Korea

*Correspondence to: *hdshin@korea.ac.kr*

Abstract — A new species, *Ramularia hydrangeicola*, causing leaf spots was observed on *Hydrangea serrata* f. *acuminata*. Morphological characteristics and phylogenetic analyses support this fungus as a distinct new species. The new fungus is described, illustrated, and compared with *R. hydrangeae*, *R. hydrangeae-macrophyllae*, and *R. philadelphi*, three additional species on hosts of the *Hydrangeaceae*.

Key words — anamorphic *Mycosphaerella*, plant pathogen

Introduction

Ramularia is an anamorph-typified genus comprising hyphomycetous asexual morphs partly associated with sexual morphs of the genus *Mycosphaerella* (*Mycosphaerellaceae*). Based on the new rules of the ICN (Art. 59) and the new "one fungus one species" principles, *Mycosphaerella* has been reduced to synonymy with *Ramularia* since the type species of *Mycosphaerella*, *M. punctiformis*, forms a *Ramularia* anamorph, *R. endophylla*, and clusters with other *Ramularia* species (Verkley et al. 2004, Braun et al. 2013). The older name *Ramularia* has priority and is now applied as holomorph name for this monophyletic unit composed of asexual and sexual morphs (Braun et al. 2013, Wijayawardene et al. 2014). Most *Ramularia* species are phytopathogenic fungi that cause leaf spots, necrosis, or chlorosis; several species are economically important pathogens. Some *Ramularia* species are saprophytic or hyperparasitic (Braun 1998). *Ramularia* species are considered host-specific, like other fungi of the *Mycosphaerellaceae*. Species of *Ramularia*

and other allied genera have conventionally been morphologically delimited according to their host plant families and genera (Braun 1995).

Since 2002, a previously undescribed leaf spot has been observed on *Hydrangea serrata* f. *acuminata* during field surveys of cercosporoid fungi in Korea. The morphological characteristics of this fungus were quite different from those of other similar hyphomycetes observed on *Hydrangea* and were similar to those of *Ramularia* as described by Braun (1998). In this study, this novel species from Korea is described and illustrated on the basis of morphological characteristics and phylogenetic analyses. This species is compared with the three *Ramularia* species previously described on hydrangeaceous hosts: *R. hydrangeae*, *R. hydrangeae-macrophyllae*, and *R. philadelphi*.

Materials & methods

Fresh material and air-dried specimens were used for morphological examinations. The fungal structures were detached from the infected leaves and mounted in a few drops of distilled water or 3% KOH on a glass slide. These slides were examined by bright field- and differential interference contrast (DIC)-light microscopy, by using an Olympus BX51 microscope (Olympus, Tokyo, Japan) for measurements and a Zeiss AX10 microscope equipped with AxioCam MRc5 (Carl Zeiss, Göttingen, Germany) for imaging. Voucher specimens were deposited in the Korea University Herbarium, Seoul, Korea (KUS).

To obtain a pure isolate, infected tissue with abundant sporulation was excised and placed in a drop of distilled water on a glass slide. A loop of conidial suspension was streaked onto 2% water agar plates supplemented with 100 mg/L of streptomycin sulfate. After 2 days of incubation at 25°C, single conidial colonies were transferred onto potato dextrose agar (PDA). Two monoconidial isolates were deposited in the Korean Agricultural Culture Collection, National Institute of Agricultural Science, Jeonju, Korea (KACC).

The fungal mycelium was harvested from the culture on PDA and genomic DNA was extracted by means of a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The internal transcribed spacer (ITS) region and the large subunit (LSU) of rDNA were amplified by using primers ITS1 and ITS4 (White et al. 1990) for ITS, and LR0R (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for LSU. The PCR amplicons were purified with the help of a QIAquick PCR purification Kit (Qiagen Inc.) and were sequenced by applying the Macrogen Sequencing Service (Macrogen, Seoul, Korea). The raw sequences were edited by means of DNASTAR version 5.05 (Lasergene, Madison, WI, USA). For phylogenetic analyses, all available ITS and LSU sequences that closely matched the extracted sequences were retrieved from the NCBI GenBank database. Sequence datasets for the two genomic loci were aligned on the base of an online version of MAFFT 7 (Katoh & Standley 2013). A phylogenetic tree was constructed by the neighbor-joining method using MEGA 6 (Tamura et al. 2013). Branches of the inferred tree were tested by a bootstrap analysis of 1000 replicates. Furthermore, a Bayesian inference analysis was performed according to MrBayes version

3.1.2 (Ronquist & Huelsenbeck 2003). Best-fit substitution models, which were obtained by applying MrModeltest version 2.2 (Nylander 2004), were used to ascertain the best nucleotide substitution model setting for each data partition. K80+I+G and GTR+I+G as nucleotide substitution models were used for ITS and LSU datasets. Branch support was calculated as posterior probability (PP) and neighbour-joining bootstrap (NJ-BS). The phylogenetic tree generated from the ITS sequence alignment was deposited in TreeBASE (http://www.treebase.org/) under accession number S17946.

Taxonomy

Ramularia hydrangeicola J.H. Park & H.D. Shin, **sp. nov.** Fig. 1

MycoBank MB813449

Differs from *Ramularia hydrangeae*, *R. hydrangeae-macrophyllae*, and *R. philadelphi* in having conspicuous, hypophyllous, pinkish or reddish tinged caespituli and longer, wider conidia that are pinkish tinged, smooth, guttulate and mostly constricted at the septa.

Type: On living leaves of *Hydrangea serrata* f. *acuminata* (Siebold & Zucc.) E.H. Wilson (*Hydrangeaceae*): Korea, Yangpyeong, Okcheon-myeon, 37°34′45″N 127°27′28″E, 18 October 2007, H.D. Shin (**Holotype**: KUS-F23039; ex-type culture, KACC43597; GenBank KT265707, KT265708)

Etymology: The epithet is derived from the host plant genus.

Leaf spots amphigenous, scattered to confluent, angular to irregular, vein-limited, distinct, small, 1–5 mm diam., or up to 10 mm when confluent, at first visible as pinkish patches on the lower surface, pale greenish on the upper surface, indistinct discolorations, later becoming brown to dark brown on both sides. Caespituli hypophyllous, conspicuous, pinkish or with reddish tinge due to abundant fungal fructification. Mycelium internal; hyphae branched, hyaline or with faint pinkish tinge, 3–5 µm wide, occasionally with superficial secondary mycelium emerging through the substomatal cavity. Stromata substomatal or sometimes intraepidermal. Conidiophores 3–8 in loose fascicles, emerging through stomata, solitary conidiophores arising from superficial hyphae, hyaline throughout or partly with pinkish tinge, 0–3-septate, simple, straight to curved, usually geniculate-sinuous, or 0–3 times geniculate near the apex, 12.5–40 × 2.5–5.0 µm. Conidiogenous cells integrated, terminal, intercalary, proliferating sympodially, 3.5–15 µm long; conidiogenous loci conspicuous, thickened and darkened, 1.2–2.1 µm diam. Conidia solitary or catenate, sometimes in short branched chains, cylindrical to obclavate-ellipsoid, hyaline or with pinkish tinge, smooth, guttulate, mostly uniseptate, occasionally 2–5-septate, constricted to non-constricted at the septa, obtuse to subobtuse at both ends, sometime gently attenuated towards each end, 18–45 × 3–7 µm; hilum minute, 1.2–1.8 µm diam., conspicuously thickened, darkened, and slightly protuberant. Colonies on PDA attaining

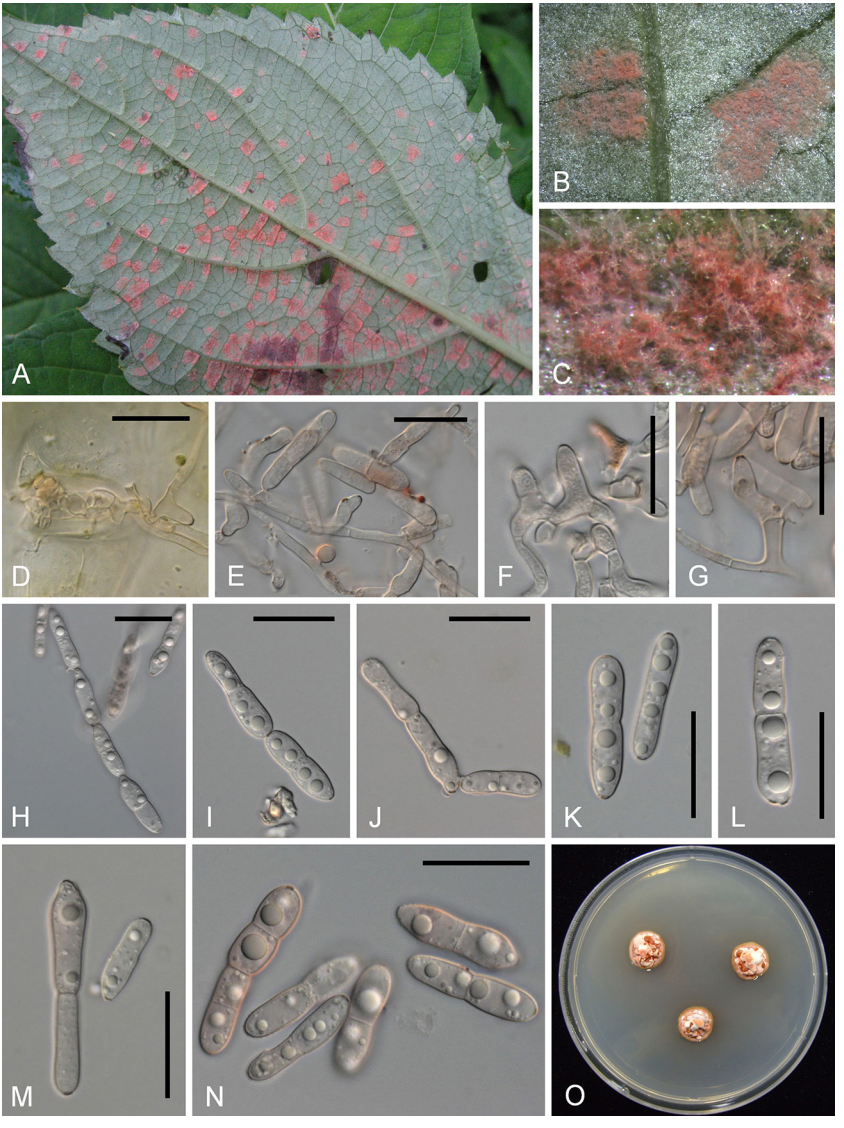

Fig. 1. *Ramularia hydrangeicola*. A: symptoms on lower leaves of *Hydrangea serrata* f. *acuminata* with distinctive traits; B, C: close-up of the leaf lesions showing pinkish caespituli; D: conidiophores emerging through the substomatal cavity; E–G: conidiophores arising from superficial hyphae; H–J: catenate conidia; K–N: conidia ranging from hyaline to reddish tinged; O: 6-week-old colonies formed on potato dextrose agar (D–N = holotype, KUS-F23039; O = KACC43597). Scale bars: D–N = 20 μm.

ca. 10 mm diam. after 6 weeks at 25°C under 12-h fluorescent light, velvety, pinkish to whitish, margins undulate, consisting of dense aerial mycelium and producing several droplets of slimy exudate. Teleomorph unknown.

Additional specimens examined: on *Hydrangea serrata* f. *acuminata*: **KOREA**, **Jeju**, 14 Sep. 2002, H.D. Shin (KUS-F19005); 2 Nov. 2007, H.D. Shin (KUS-F23141); **Pyeongchang**, 27 Sep. 2002, H.D. Shin (KUS-F19080); 20 Sep. 2003, H.D. Shin (KUS-F19700); 9 Oct. 2008, H.D. Shin (KUS-F23778; culture, KACC44254; GenBank KT265709, KT265710); **Yangpyeong**, 28 Aug. 2008, H.D. Shin (KUS-F23614); **Chuncheon**, 7 Oct. 2008, H.D. Shin (KUS-F23759); **Gapyeong**, 13 Oct. 2011, H.D. Shin & J.H. Park (KUS-F26314); **Wonju**, 18 Sep. 2014, H.D. Shin & J.H. Park (KUS-F28177).

Results & discussion

Several mycosphaerellaceous fungi have been described on *Hydrangea* species, including *Cercospora*, *Pseudocercospora*, *Passalora*, *Phacellium*, and *Ramularia* spp. (Braun 1998, Crous & Braun 2003, Braun & Hill 2008, Farr & Rossman 2015). Our new species, *R. hydrangeicola*, is characterized by having mostly uniseptate conidia with thickened, darkened hila, either formed solitary or catenate, ranging from cylindrical to obclavate-ellipsoidal in shape and from hyaline to reddish tinged in color. These characteristics are consistent with the main characteristics of *Ramularia* (Braun 1998).

Three *Ramularia* species have been recorded on hosts of the family *Hydrangeaceae: R. hydrangeae* Y.L. Guo & U. Braun was described on *H. bretschneideri* from China (Braun 1998), based on material originally misdetermined as *R. saxifragae* (Guo 1993); *R. hydrangeae-macrophyllae* U. Braun & C.F. Hill was described on *H. macrophylla* from New Zealand (Braun & Hill 2008); and *H. philadelphi* Sacc., originally described from Italy, has been recorded on *Philadelphus* spp. from Europe, North America, and Asia (Braun 1998, Farr & Rossman 2015).

Ramularia hydrangeae differs from *R. hydrangeicola* in having inconspicuous, greyish white caespituli and hyaline, verruculose, shorter (8–35 µm), and narrower (2–5 µm) conidia (Braun 1998); *R. hydrangeae-macrophyllae* differs in having inconspicuous caespituli and shorter (4–18 µm), narrower (1.5–2.5 µm), and 0–1-septate conidia (Braun & Hill 2008); and *R. philadelphi* differs in having amphigenous, greyish white caespituli and hyaline, verruculose, shorter (8–20 µm), and narrower (2.5–5.0 µm) conidia (Braun 1998).

Phacellium hydrangeacearum U. Braun on *Jamesia americana* from the USA is another ramularioid fungus on a hydrangeaceous host, but easily distinguished by its synnematous conidiomata and verruculose conidia (Braun 1998).

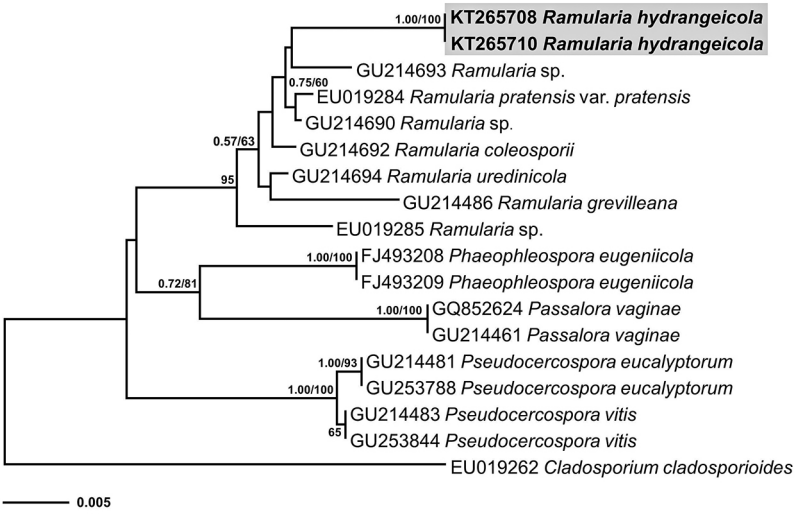

Fig. 2. LSU rDNA phylogenetic tree generated by a neighbor-joining method by using MEGA6 with 1000 bootstrap replications based on sequences of *Ramularia* and allied genera. Branch support is indicated above the branches (PP/NJ-BS). Sequences of *R. hydrangeicola* are shown in bold and their well-supported clade is highlighted in gray.

The holotype and paratype specimens of *R. hydrangeicola* yielded 1172-bp LSU and 495-bp ITS sequences. The LSU sequences were congruent between the two isolates, whereas their ITS sequences differed by three base-pairs. A GenBank BLAST search revealed that the LSU sequences had 99% similarity to GU214693 (*Ramularia* sp.), GU214690 (*Ramularia* sp.), and GU214692 (*R. coleosporii*); and the ITS sequences had 94% similarity to GU214693 (*Ramularia* sp.), EU019284 (*R. pratensis*), and KJ504787 (*R. decipiens*). Based on the molecular phylogenetic trees inferred from the neighbor-joining method and Bayesian analyses using ITS and LSU sequences, *R. hydrangeicola* formed an independent clade with high bootstrap values: PP = 1, NJ-BS = 100% (Figs 2, 3). Moreover, the LSU phylogeny indicated that this species belongs in the genus *Ramularia*, and the ITS phylogeny revealed that it forms a separate lineage among *Ramularia* species. Although there are no comparable sequence data for *R. hydrangeae*, *R. hydrangeae-macrophyllae*, and *R. philadelphi*, *R. hydrangeicola* clearly differs morphologically from these species. Therefore, *R. hydrangeicola* is recognized as a new species based on the present morphological and molecular data.

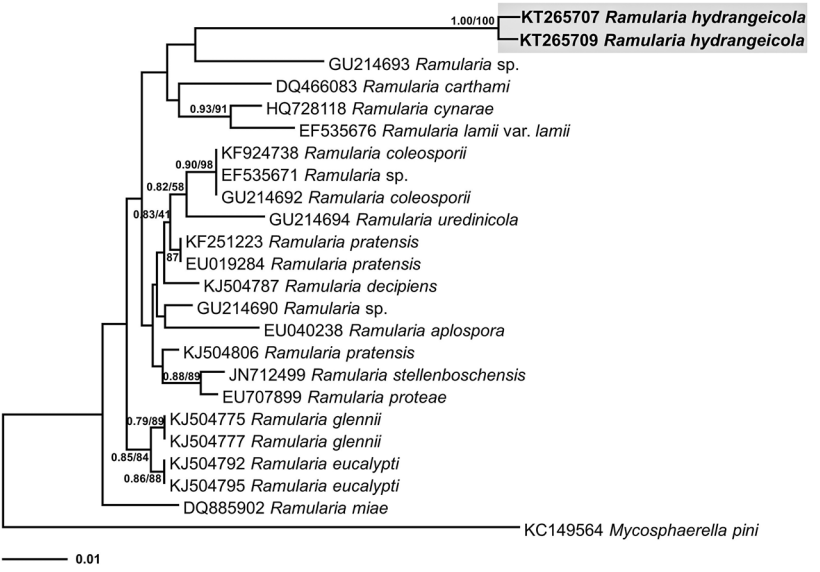

Fig. 3. ITS rDNA phylogenetic tree generated by a neighbor-joining method by using MEGA6 with 1000 bootstrap replications based on sequences among *Ramularia* species in GenBank database. Branch support is indicated above the branches (PP/NJ-BS). Sequences of *R. hydrangeicola* are shown in bold and their well-supported clade is highlighted in gray.

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Literature cited

Braun U. 1995. A monograph of *Cercosporella*, *Ramularia* and allied genera (phytopathogenic hyphomycetes). Vol. 1. IHW-Verlag, München.

Braun U. 1998. A monograph of *Cercosporella*, *Ramularia* and allied genera (phytopathogenic hyphomycetes). Vol. 2. IHW-Verlag, München.

Braun U, Hill CF. 2008. New species and new records of foliicolous hyphomycetes from New Zealand. Australasian Mycologist 27: 45–56.

Braun U, Nakashima C, Crous PW. 2013. Cercosporoid fungi (*Mycosphaerellaceae*) 1. Species on other fungi, *Pteridophyta* and *Gymnospermae*. IMA Fungus 4: 265–345. http://dx.doi.org/10.5598/imafungus.2013.04.02.12

Crous PW, Braun U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodiversity Series 1: 571 p.

Farr DF, Rossman AY. 2015. Fungal databases. Syst. Mycol. Microbiol. Lab., Online publication, ARS, USDA. Retrieved July 1, 2015.

Guo YL. 1993. Foliicolous hyphomycetes of Xiaowutai in Hebei Province. Mycosystema 6: 91–102.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30: 772–780. http://dx.doi.org/10.1093/molbev/mst010

Nylander JAA. 2004. MrModeltest version 2.0. Program distributed by the author. Uppsala University, Uppsala, Sweden.

Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycol. Res. 98: 625–634. http://dx.doi.org/10.1016/s0953-7562(09)80409-7

Ronquist F, Huelsenbeck JP. 2003, MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. http://dx.doi.org/10.1093/bioinformatics/btg180

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729. http://dx.doi.org/10.1093/molbev/mst197

Verkley GJM, Crous PW, Groenewald JZ, Braun U. 2004. *Mycosphaerella punctiformis* revisited: morphology, phylogeny, and epitypification of the type species of the genus *Mycosphaerella* (*Dothideales, Ascomycota*). Mycol. Res. 108: 1271–1282. http://dx.doi.org/10.1017/s0953756204001054

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172: 4238–4246.

White TJ, Bruns T, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis et al. (eds.), PCR protocols: a guide to methods and applications. San Diego, Academic Press. http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1

Wijayawardene NN, Crous PW, Kirk PM et al. 2014. Naming and outline of *Dothideomycetes* – 2014 including proposals for the protection or suppression of generic names. Fungal Divers. 69: 1–55. http://dx.doi.org/10.1007/s13225-014-0309-2